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16. ABSTRACT

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IS DELAYED NEUROTOXICITY A PROPERTY OF ALL ORGANOPHOSPHORUS COMPOUNDS? A STUDY WITH A MODEL COMPOUND: PARATHION*

SALAH A. SOLIMAN**, JACKIE FARMER and AUGUST CURLEY

Neurotoxicology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711 (U.S.A.)

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SUMMARY

A recently reported hypothesis of other investigators that the induction of delayed neurotoxicity is a property of all organophosphorus compounds including parathion was evaluated in light of the inability of parathion to induce in our laboratory any clinical, histological, or biochemical signs of delayed neurotoxicity in hens following a very intensive dosing regimen. Parathion was administered orally or applied dermally as 1 mg/kg/day for 1 week and then the dose was increased by 1 mg/kg/day at weekly intervals up to 6 mg/kg/day which was given thereafter until a total of 90 doses. Results indicate that parathion either orally or dermally did not produce delayed neurotoxicity in hens comparable to that induced by tri-*ortho*-cresyl phosphate (TOCP) in this experiment. This finding is supported by clinical, histological, and biochemical evidences. No clinical signs or histopathological changes in spinal cords and sciatic nerves of the type associated with delayed neurotoxicity were observed in any of the surviving parathion-treated hens. Moreover, this extensive treatment with parathion resulted in no significant in vivo effect on neurotoxic esterase, an esterase believed to be the initial target in the genesis of delayed neurotoxicity. These results

*This paper has been reviewed by the Health Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

**Visiting Scientist, Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

Abbreviations: DEF, tributyl phosphorotrithioate; EPN, ethyl *p*-nitrophenyl phenylphosphonothioate; GLC, gas liquid chromatography; H&E, hematoxylin and eosin; LFB, Luxol fast blue; NTE, neurotoxic esterase; OP, organophosphorus; PAM, 2-pyridine aldoxime methyl chloride; PV, phenyl valerate; SDS, sodium dodecyl sulfate; TOCP, tri-*ortho*-cresyl phosphate.

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agree with the general hypothesis that delayed neurotoxicity is a special toxic effect of some but not all of the organophosphorus esters.

INTRODUCTION

Delayed neurotoxicity is one of the most serious toxic effects of some organophosphorus esters. This effect is characterized pathologically by distal axonal degeneration and secondary demyelination of both the central and peripheral nerve axons and by a time-delay period of from 8 to 14 days before onset of clinical and pathologic signs after exposure to the neurotoxic agent [1,2]. The primary biochemical event has been suggested to be phosphorylation of a nervous system protein referred to as neurotoxic esterase (NTE). A second time-dependent reaction involving transfer of a side-chain from the phosphoryl group to some other site (aging) must also occur [3–7].

Many reviews on the toxic neuropathic effect that is induced by some organophosphorus compounds have been published [4,5,8–12]. The reference articles in those reviews were in agreement with the general hypothesis that some, but not all, of the organophosphorus derivatives induced toxic neuropathy. Graham et al., found in in vitro studies that merphos, tributyl phosphorotrithioate (DEF), ethyl *p*-nitrophenyl phenylphosphonothioate (EPN), parathion, TOCP, and leptophos, respectively, caused significant reductions in cocaine-inhibitable [^{3}H]norepinephrine uptake in differentiating human neuroblastoma cells in tissue culture [13]. They concluded that these findings were consistent with the hypothesis that delayed neurotoxicity was a property of all organophosphorus compounds. This hypothesis was reiterated in a recent review [14].

Generally, parathion, *O,O*-diethyl-*O*(4-nitrophenyl)phosphorothioate, has been used as a negative control compound in studies evaluating or elucidating the delayed toxic neuropathic action of organophosphate esters [15–19]. Paraoxon, the active metabolite of parathion, is used to inhibit esterases but not NTE in the technique of assaying NTE activity [20,21]. Paraoxon, in the mechanistic studies of NTE, is an inhibitor that is used to distinguish this enzymic activity. In this present work, we decided to reinvestigate whether parathion was neurotoxic, particularly since it had recently been advocated that all organophosphorus compounds would produce delayed neurotoxicity. Study was conducted to test if parathion as a model organophosphorus ester produces any clinical, histopathological or biochemical signs of delayed toxic neuropathy when administered to chickens in a dosing regimen that allows buildup of parathion to a maximal tolerated dose without the protection of antidotes and continuation of that dose for 90 days.

MATERIALS AND METHODS

Parathion, *O,O*-diethyl-*O*-(4-nitrophenyl)phosphorothioate (98%, gas

liquid chromatography (GLC)), and its oxygen analogue, paraoxon (97%, GLC), were obtained from Chemical Service Co. and Aldrich Chemical Co., respectively. Tri-*ortho*-cresyl phosphate (TOCP) (99%, GLC), was obtained from Eastman Kodak Company. Mipafox and phenyl valerate (PV) were synthesized as previously reported [20,22]. All the other chemicals used were of the highest purity grade available from Sigma and/or Aldrich Chemical Companies.

Treatment and care of chickens

Adult white leghorn hens (1.5—2.0 kg) were obtained from Featherdown Farm, Apex, NC. Hens were divided into 5 groups of 10 birds each and allowed to adjust to the experimental conditions for 2 weeks before treatment. The hens were supplied with food and water ad libitum during this experiment. The first group of hens was treated orally with corn oil at 1 ml/kg/day for 90 days and kept as vehicle control group for the oral treatments. The second group was treated dermally with acetone at 0.1 ml/kg/day for 90 days and kept as vehicle control group for the dermal treatments. The third group of chickens was treated orally with TOCP at 500 mg/kg once and kept as positive control group with respect to the delayed neurotoxic syndrome. The fourth and fifth groups of hens were treated orally and dermally, respectively, with parathion at 1 mg/kg/day for a week and then at 2 mg/kg/day during the second week, 3 mg/kg/day during the third week, 4 mg/kg/day during the fourth week, 5 mg/kg/day during the fifth week, and 6 mg/kg/day afterwards to complete 90 doses. Parathion was dissolved in corn oil and administered as 1 ml/kg for the oral treatment. It was dissolved in acetone and applied on the combs of hens as 0.1 ml/kg for the dermal treatments. The dosing regimen was carried out 7 days a week. Changes in the hens' weights were recorded weekly during the experiment and the doses were recalculated to meet with these weekly changes.

Clinical observations

The hens were observed each day before the daily treatment throughout the experiment for any abnormalities and for signs of delayed toxic neuropathy such as leg weakness, ataxia, and paralysis. The symptoms were graded on an 8-point scale comparable to that of Cavanagh et al., and Davies et al. [23,24]. Scale and definitions are presented in Table I.

Four of the TOCP-treated hens were killed by decapitation 24 h after treatment and the brains were taken for the biochemical assay. The remaining TOCP-treated hens were killed by decapitation at the time each hen became paralyzed (from 20 days to 30 days after treatment). All of the remaining chickens of groups 1, 2, 4, and 5 were killed by decapitation 1 day following the last treatment. Brains of the killed birds were taken for the biochemical assay, and the spinal cords and sciatic nerves were fixed in 10% neutral buffered formalin and submitted for histopathologic study.

Histologic examination

After fixation of the spinal cords and sciatic nerves, paraffin sections

TABLE I

DEFINITION AND DEGREE OF DELAYED NEUROTOXICITY USED TO CHARACTERIZE HENS

Definition	Degree of neuropathy (Points)	Description
Normal	0	Normal hens able to run across a cement floor. These hens frequently stood on one foot and rarely flew around.
Clumsiness	1—2	Hens with definite incoordination and they tended to fly rather than run if disturbed. These hens stumbled when running on cement floor and rarely stood on one foot.
Moderate ataxia	3—4	Hens preferred to squat but able to walk for few steps if they were pushed gently.
Marked or severe ataxia	5—6	Hens with inability to maintain an upright stand for any length of time. These hens progressing on their hock.
Paralysis	7—8	Hens with total inability to rise or move from their position

were prepared for light microscopy. Cross and longitudinal sections of the spinal cord (cervical, thoracic, and lumbar levels) and longitudinal sections of the sciatic nerves were stained with luxol fast blue (LFB) combined with hematoxylin and eosin (H&E) or by the Holme's silver method.

Assay of NTE and AChE activities

Hen brain microsomal fractions were obtained as reported previously [22]. The NTE activity in the hen brain microsomal preparations was determined using the differential technique [20] with slight modifications. Paired samples of the microsomal preparations, each of 200 μl, were preincubated in Tris—HCl buffer (50 mM, pH 8) containing 0.2 mM EDTA for 20 min with paraoxon (40 μM) plus either (a) buffer or (b) mipafox (50 μM) in a final volume of 3 ml at 37°C. After preincubation, 20 μl of a solution of PV in dimethyl formamide (25 mg/ml) was added and the incubation was continued for 10 min. The enzymatic reaction was stopped at the end of the incubation period by adding 2 ml of sodium dodecyl sulfate (SDS) solution (1% w/v) in the Tris/EDTA buffer containing 4-aminoantipyrine (0.025% w/v). Tube contents were mixed immediately after addition of SDS, 1 ml of aqueous potassium ferricyanide solution (0.4% w/v) was then added. Absorbance of the resultant stable red color was recorded at 510 nm after 10 min against blanks from which PV was omitted. NTE activity was calculated mathematically using this technique by subtraction of the remaining PV-hydrolyzing activity in tube (b) from that in tube (a). The protein contents of the preparations were determined using the method of Lowry [25].

Hen brain microsomal AChE activity was determined using the Ellman method [26].

RESULTS

Figure 1 presents the bi-weekly changes in the parathion-treated hens' body weights during this experiment. Treatment with parathion either orally or dermally resulted in severe or significant loss of hen body weight,

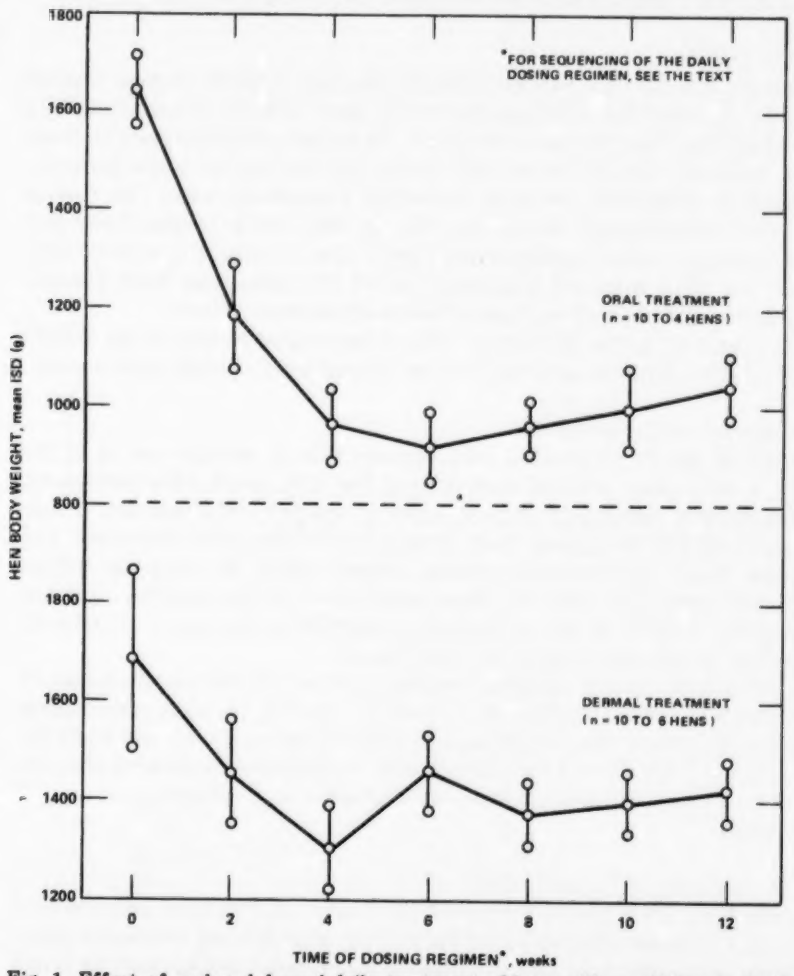


Fig. 1. Effect of oral and dermal daily treatment of hens with parathion on the hen body weight. *Hens were treated daily with parathion at 1 mg/kg for a week and then the dose was increased by 1 mg/kg/day at weekly intervals up to 6 mg/kg/day which was given thereafter for a total of 90 doses.

respectively, as the dose level was increased. The decrease of hen body weight due to treatment with parathion orally and dermally reached minimum peaks after 4–6 weeks of treatment after which the surviving treated hens slightly gained weight. However, the orally and dermally parathion-treated surviving hens were about 63% and 85% of their corresponding average weight before treatment. Corn oil- and acetone-treated control hens appeared to have no significant change in their body weight during this experiment and their average weight was 1668 ± 139 g. No significant change in weight of TOCP-treated hens was recorded until they were killed.

Cholinergic effects

Cholinergic effects were observed during the first 5 weeks in hens treated either orally or dermally with parathion and these effects, as manifested by salivation and diarrhea, became more severe as dosage level increased. In these 2 groups, paralysis seemed to develop from time to time in some hens but the apparently paralyzed chickens recovered completely when the dosing regimen was discontinued for a day. Six of the orally treated hens and 4 of the dermally treated group were found dead during the experiment. Following the fifth week of treatment, all of the remaining hens became normally active and showed no signs of acute cholinergic effects.

No symptoms of acute cholinergic effects developed in any of the TOCP-treated hens. The corn oil- and the acetone-treated control hens were normal.

Delayed neurotoxicity assessment

Although all the TOCP-treated hens appeared to be normal and as active as the corn oil-treated control hens during the first week after treatment, they subsequently developed clinical signs of delayed neurotoxicity. These clinical signs started to appear from 7 days to 12 days after treatment and to progress from leg weakness, ataxia, severe ataxia to paralysis within 30 days after treatment. On the other hand, none of the parathion-treated hens, whether treated orally or dermally, developed any signs of delayed neurotoxicity at any time during the experiment.

To confirm the clinical findings, central and peripheral nerve tissues of the sacrificed parathion-treated hens were subjected to histopathological examination as well as the corresponding tissue from corn oil- and acetone-treated control hens. This histopathological examination indicated that no degenerative lesions of axons and myelin sheaths were observed in any of the specimens.

Effects on hen brain NTE and AChE

The remaining NTE and AChE activities in brain microsomal preparations from the surviving parathion-treated hens 1 day after the last treatment were determined. Also, the activities of these enzymes were determined in brain microsomal preparations of 4 hens of the TOCP-treated birds killed 1 day after the single treatment. Table II presents the in vivo effects of parathion, orally and dermally, and TOCP, orally, on the hen brain microsomal NTE

TABLE II

IN VIVO EFFECTS OF PARATHION AND TOCP ON HEN BRAIN MICROSOMAL NEUROTOXIC ESTERASE (NTE) AND ACETYLCHOLINESTERASE (AChE) ACTIVITIES

Treatment and number of hens used	Route	Regimen (mg/kg × days)	% Remaining activity[a]	
			NTE[b]	AChE[c]
Parathion (4)	Oral	1 up to 5 × 7 then 6 × 55	86.4 ± 11.7	11.6 ± 4.3
Parathion (6)	Dermal	1 up to 5 × 7 then 6 × 55	87.5 ± 14.2	18.2 ± 4.7
TOCP (4)	Oral	500 × 1	8.6 ± 2.2	89.8 ± 3.9

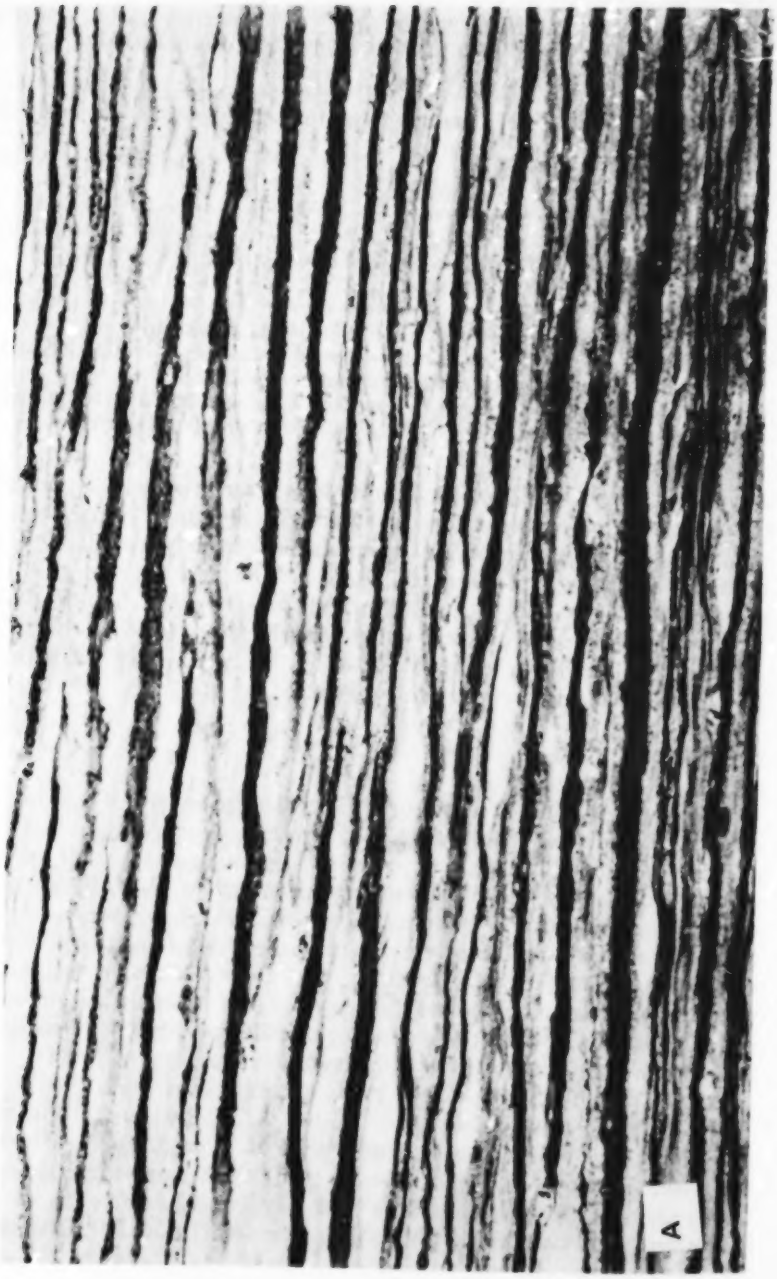
[a]Percent remaining activity relative to the corresponding controls.
[b]NTE activity of corn oil- and acetone-treated hens was 56.14 ± 1.89 and 54.72 ± 1.39 nmol of phenol produced/min/mg protein, respectively.
[c]AChE activity of these 2 control groups of hens was 105.3 ± 14.05 and 114.7 ± 10.21 nmol of acetylthiocholine hydrolyzed/min/mg protein, respectively.

and AChE activities. The enzymic activities are reported as percentages of the remaining activities (mean ± standard deviation). Neurotoxic esterase activities in brain microsomal preparations from corn oil- and acetone-treated hens were equivalent to 56.14 ± 1.89 and 54.72 ± 1.39 nmol of phenol produced/min/mg protein, respectively, using 6 individual brains from each group. The corresponding AChE activities in the same preparations were 105.30 ± 14.05 and 114.71 ± 10.21 nmol of acetylthiocholine hydrolyzed/min/mg protein, respectively.

DISCUSSION

Neurotoxic organophosphorus (OP) compounds have been reported to react with a specific protein, NTE, and subsequently the phosphorylated protein undergoes "aging" in which a side-chain is split off of the phosphoryl group [3–7]. Fundamentally important in the differential method of assaying for NTE activity is to distinguish this enzymic activity from other phenyl valerate-hydrolyzing esterases by its insensitivity to paraoxon, the active metabolite of parathion, and by its sensitive response to the selective inhibitor mipafox or the oxygen analogues of the neurotoxic OP esters [3,20,27]. Parathion has been generally accepted as a negative control for screening OP compounds for delayed neurotoxicity [15–19].

The results obtained in this study indicate that parathion, at repeated doses of 1 mg/kg/day for 1 week; then of 2, 3, 4 and 5 mg/kg/day for 5 consecutive weeks, respectively; and of 6 mg/kg/day afterwards for 90 doses did not produce any signs of delayed neurotoxicity in the hen. Under these test conditions, there were no clinical, histological, or biochemical evidence of delayed neurotoxicity (Tables II and III). The chickens in this experiment received, cumulatively, about 100 times the LD_{50} values for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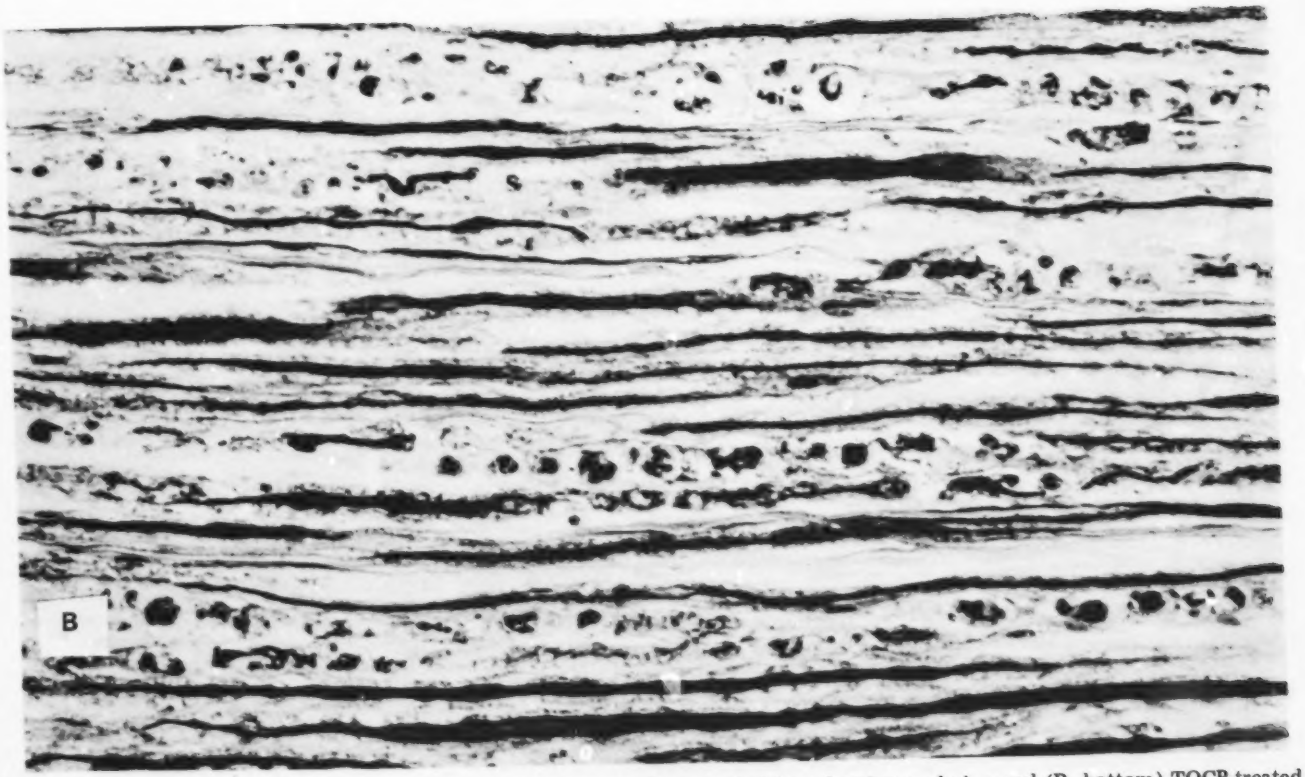

Fig. 2. Longitudinal sections of sciatic nerve of (A, top) parathion-treated hen showing no lesion and (B, bottom) TOCP-treated hen showing fragmented degenerated axons. Holme's/Lux-1 Fast Blue stains, × 200.

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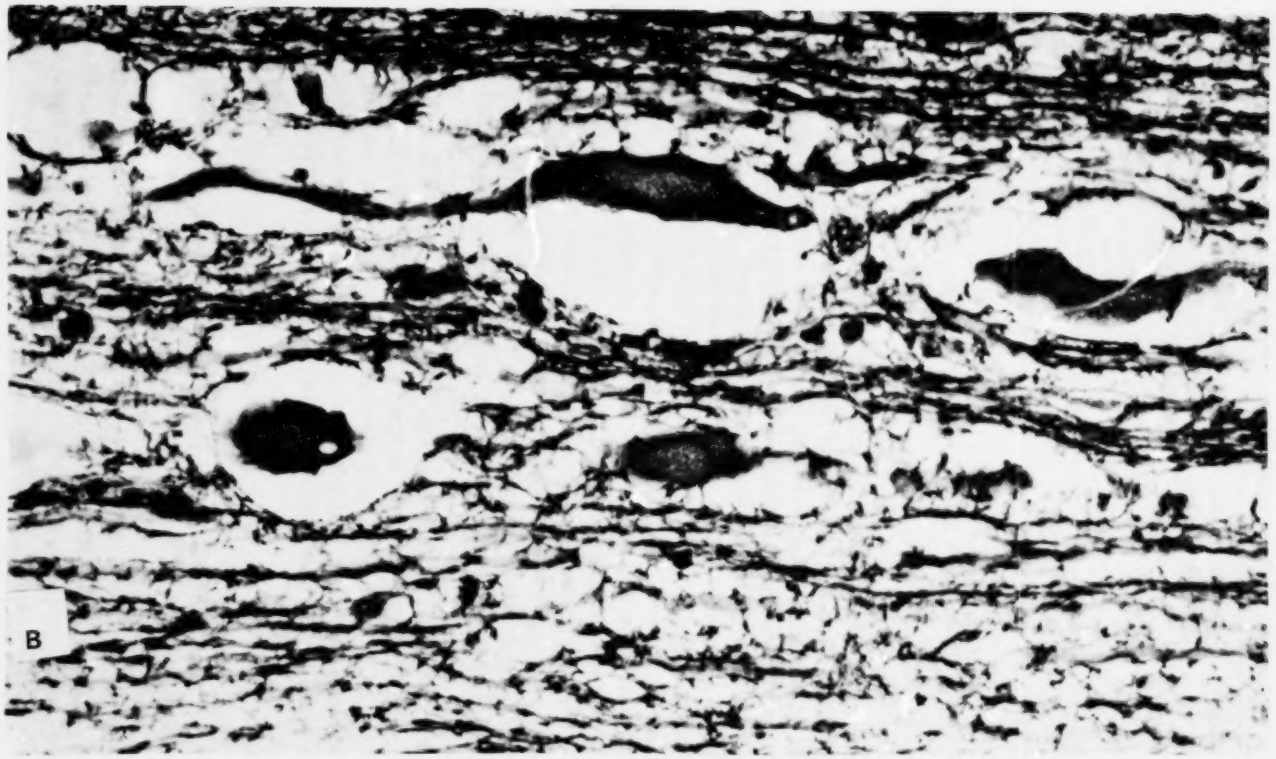

Fig. 3. Longitudinal sections of spinal cord of (A, top) a parathion-treated hen showing no lesion and of (B, bottom) a TOCP-treated hen showing details of degenerated axons. Luxol Fast Blue/Hematoxylin and Eosin stain, × 200.

TABLE III

CLINICAL, HISTOLOGIC AND BIOCHEMICAL EFFECTS OF PARATHION IN HENS

Treatment	Route	Dosing regimen (mg/kg × days)	Cumulative dose (mg/kg)	Number of survived hens	Signs of delayed neurotoxicity at time of necropsy	
					Clinical	Histological
Corn oil	Oral	1 (ml) × 90	90 (ml)	10	(−)	(−)
Parathion	Oral	1 up to 5 × 7 then 6 × 55	435	4	(−)	(−)
Acetone	Dermal	0.1 (ml) × 90	9 (ml)	10	(−)	(−)
Parathion	Dermal	1 up to 5 × 7 then 6 × 55	435	6	(−)	(−)

parathion without treatment with antidotes such as atropine sulfate and 2-pyridine adoxime methyl chloride (PAM). Figures 2 and 3 present photomicrographs of longitudinal sections of sciatic nerve and spinal cord, respectively, from parathion- and TOCP-treated hens. TOCP photomicrographs present fragmentation of swollen, degenerate, and necrotic axons while none of these kinds of abnormal histologic changes were shown in the parathion-treated chickens (Figs. 2 and 3). The clinical studies indicate that no morphological symptoms of delayed neurotoxicity developed in any of the parathion-treated hens and no histopathological changes of the type associated with delayed neurotoxicity were observed in any of the spinal cord or sciatic nerve specimens taken from the surviving parathion-treated hens. These results are in agreement with those reported previously that parathion at 1.0 mg/kg/day for 90—120 days did not produce either clinical or pathological signs of delayed neurotoxicity in hens [15,16]. The biochemical results indicate that treatment of the hens with parathion either orally or dermally produces no significant in vivo effect on brain NTE activity but parathion inhibits more than 80% of the AChE activity 1 day following the last treatment. TOCP, which is a known inducer of delayed neurotoxicity, produces about 90% in vivo inhibition of hen brain NTE activity and only about 10% inhibition of AChE activity 1 day following treatment.

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